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STUDIES ON SIMPLE AND DIFFERENTIAL METHODS OF STAINING ENCAPSULATED PNEUMOCOCCI IN SMEAR AND SECTION.*

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IN the course of some experimental studies on pneumococcus infection, the technic of previous observers recommended for the staining of the encapsulated organisms was tested, and various new methods were devised in the hope of securing reliable procedures by which pneumococci may be differentially stained in cover-glass preparations and in sections of tissue.

My experience with these methods, old and new, it is the purpose of this paper to record.

PREVIOUS METHODS.

Simple staining.—In the simple† routine staining with aqueous-gentian-violet or carbol fuchsin, smears of pneumococcus exudates may show the organisms encapsulated; but this is extremely uncertain. Similarly, under favorable conditions many of the older special methods‡ devised for capsule staining often give excellent preparations, but the results vary, and are therefore unreliable when compared with those obtained with the simple, perfected technic used by recent observers. The most reliable and practical of all these methods in my experience are based wholly or in part on principles first adopted by Guarnieri.

In 1888 Guarnieri[5] made determinations of the solubility of the pneumococcus capsule in acid, alkaline, and neutral salt solutions; and finally he obtained the proteid reaction with Millon's reagent, thus indicating an albuminous composition. On these determinations Guarnieri devised a new method of staining the encapsulated organisms in exudates: smears fixed in the flame

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†Pane.[12]

‡Friedländer,[3] Ribbert,[13] Roux,[14] Muir,[11] MacConkey,[9] Gordon,[4] Kolle and Wasserman.[8]

were stained with analin-gentian-violet, then washed and differentiated in 2 per cent aqueous sodium chloride, rewashed very quickly in water, dried, and mounted in balsam. Similar methods have since been recommended. Thus, Welch[16] adopted Guarnieri's method of staining, but mounted the specimen in the salt solution, and suggested a preliminary treatment with glacial acetic acid on the ground that the capsule was composed of mucin.*

Buerger[2] also adopted Guarnieri's method, but recommended a preliminary fixation of the capsule, first in a solution of chromic acid and bichloride of mercury, then in an alcoholic solution of iodine (U. S. P.). With Gram's method of staining this fixation is essential, but in the simple procedures the advantage of it is less apparent, for practically the same results are secured with Guarnieri's less complicated method.

Hiss,[6] however, by substituting the ordinary aqueous-gentian-violet stain for the unstable anilin-gentian-violet, and by using 0.25 per cent potassium carbonate solution, which to some extent clears the field, simplified and improved materially the technic of capsule staining. Finally, by using a 20 per cent copper sulphate wash instead of the potassium carbonate he found that the specimen could be dried and mounted in balsam.

Formerly capsules were found only in the exudates of infected animals, but now they are readily demonstrated in organisms growing in artificial media. Boni found that when cultures of pneumococci are smeared in egg albumin, the capsules are easily stained. Hiss secured similar results with blood serum, and by means of his more reliable methods of staining was able to determine more fully the importance of utilizing this principle in the morphological study of the pneumococcus.

By virtue of these modern procedures it is now a comparatively simple matter to demonstrate capsules on these organisms. It is no longer a question of how encapsulated pneumococci may be stained, but of how they may be most simply and reliably stained.

*Welch does not state the reasons for this belief; it is therefore difficult to refute the positive, though ncomplete, observations of Guarnieri. Mucin is a glycoproteid and reacts to Millon's reagent, but the solubilities differ, and capsules are more constantly noted in albuminous media than in the presence of mucin. In fact, the mucous secretions of the mouth are not a particularly favorable environment for the demonstration of capsules, whereas capsules are readily obtained in simple broth, if only the albumin or even the peptones be sufficiently increased.

The methods of Guarnieri, of Welch, and of Buerger, reliable, though in one way or another complicated, all give temporary mounts, and are thus unsatisfactory; particularly when similar or better results may be secured by simpler procedures giving permanent preparations in balsam, which may be kept for future reference, as with the copper sulphate method of Hiss.[6]

Differential staining.—These simple methods of staining encapsulated pneumococci, however, do not always suffice to differentiate the pneumococcus from other capsule-forming bacteria.* To complete this differentiation further steps are necessary. In pneumonic exudates, pneumococci, streptococci, and pneumobacilli (Friedländer) are frequently associated together. The streptococci, except possibly under especially favorable conditions, rarely show capsules. The pneumobacilli not only are encapsulated, but the short and degenerating forms are practically indistinguishable from the pneumococci. The pneumobacillus, however, decolorizes by the Gram stain; but this does not show capsules, and when these contaminated exudates are examined for the morphological determination of the presence of these species, both the capsule and the Gram stains are required, and even then the observations may not be accurately correlated. Cultural characters are more precise and final, but require time; and, furthermore, the streptococci and pneumobacilli, when present, usually predominate; they also grow so rapidly, and the pneumococci are frequently in such small numbers, that, unless exceptional precautions are taken, the pneumococci may not be found.

For these reasons efforts have been made to obtain a reliable method which would demonstrate the capsules and the Gram differential in the same preparation. This result has been noted in specimens overexposed, or exposed with heat to the anilin stain and the iodine solutions; but this is so exceptional that special methods have been devised.

For this purpose Smith[15] fixes the smears of fresh sputum from pneumonia patients in the usual way by heat. The films are then steamed in anilin-gentian-violet and in the iodine solution. After the usual alcohol decolorization and a few seconds' exposure to a mixture of alcohol, 4 parts, ether, 6 parts, the smear is counterstained, first in aqueous eosin, then in Loeffler's methylene blue. This is followed by slight decolorization in 95 per cent alcohol, dehydration in absolute alcohol, xylol, and balsam.

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*The morphological differences in the capsules of the pneumococci, as compared with other encapsulated organisms resembling the pneumococcus, which Buerger obtained with his simple stain, and upon which he lays so much stress, depend chiefly upon the varying stages of development or degeneration and solution of the capsule, and upon the degree of decolorization. They are in no sense differential.

Buerger[2] fixes the smears of encapsulated pneumococci in Müller's fluid saturated with bichloride, for one-half minute,* and washes in water. After one minute's exposure to an alcoholic solution of iodine (U. S. P.), and washing in alcohol, the smear is stained in the usual way by the Gram method—anilin-gentian-violet, Lugol's solution, alcohol decolorization—and washed in water. After counterstaining in aqueous fuchsin, the preparations are washed and examined in 2 per cent salt solution.

With these methods of Smith and of Buerger the bacterial cells, under favorable conditions, may be stained by the Gram stain and demonstrated with capsules. The procedures, however, are complicated, not as accurate and reliable as the simple capsule stains, or give temporary mounts; and none of the methods thus far considered are applicable to the demonstration of encapsulated organisms in sections of diseased tissues.

THE NEW METHODS.

In the attempt to secure methods by which the differential staining of encapsulated organisms in tissues as well as films could be easily accomplished with a reasonable degree of certainty, a great variety of procedures were tried without success before satisfactory results were finally obtained. Suffice it to note that all the efforts to keep the capsules from dissolving in the course of hardening, imbedding, and staining, by the addition of various chemicals to the solutions, so successfully adopted in the simple methods of Guarnieri and of Hiss for films, failed to give reliable results. Exceptionally, a few encapsulated organisms were stained in sections treated in this fashion, but the result of these procedures proved to be wholly beyond control, and was thus of no practical value. It was therefore evident, if the Gram stain was to be used, that the solution of the problem depended primarily upon securing a permanent fixation or coagulation of the capsule. Obviously, success in securing permanent fixation depended upon the composition of the capsule. In the absence of data to the contrary, the results of Guarnieri's chemical studies, and the fact that capsule preservation or formation is largely determined by the presence of albumin, suggested with reasonable probability an albuminous composition. Attention was therefore directed to the study of the action of bichloride of mercury and formalin, which not only precipitate albumins, but enter into chemical combination with them.

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* In my experience with this method longer exposures are advisable; in fact, essential.

Bichloride methods.—Bichloride of mercury was tested in both aqueous and alcoholic solutions. Alcoholic solutions proved more efficient. Dried smears, fixed in saturated bichloride-alcohol for a few minutes and washed in water, may be stained in aqueous-gentian-violet, and mounted in the usual way, or they may be decolorized by retreatment in the bichloride-alcohol for a few seconds before washing in water and drying. In these preparations the encapsulated cells are often very sharply demonstrated in a perfectly clear field. With the Gram differential procedures the capsules fixed in bichloride-alcohol rarely* withstand the osmosis required to bring the specimen to a balsam mount, and as applied to the study of tissues in section it was a complete failure.

Bichloride-alcohol thus proved valuable in coagulating and partially fixing the capsules, and also in clearing the field; but, aside from this, it offered little practical advantage over the simple staining procedures of other observers.

Formalin methods.—The fixation obtained with strong solutions of formalin proved more stable. It was also found that, when desirable, formalin may be effectively used as a wash after simple staining with aqueous-gentian-violet, or 5 to 10 per cent may be added to the stain to shorten the procedure. Although useful and simple, these methods offer no special advantage over the definite fixation secured by the stronger (40 per cent) solutions of formalin,† after which a great variety of staining as procedures, differential well as simple, may be readily adopted according to the character of the material to be examined, and the purposes of the examination. The simple routine gentian-violet stain may be used, or this, after drying, may be decolorized for a few seconds in saturated bichloride-alcohol and counterstained, or the Gram differential method may be employed. Finally bits of diseased tissue may be hardened in 40 per cent formalin, imbedded in celloidin, and sections cut and stained by simple and differential methods to demonstrate the presence of encapsulated organisms.

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*Excellent balsam preparations for demonstration purposes were readily secured, but for routine work the results were uncertain. With other species of encapsulated bacteria this may prove more reliable for in sections of tissues hardened in bichloride Wright and Mallory[17] demonstrated capsules on a Gram negative organism resembling the Friedländer bacillus.

†The addition of alkali to the formalin interfered seriously with the fixation, but the addition of acetic acid 1 to 2 per cent in some instances proved advantageous, especially in securing penetration of the tissues, and also it was thought, by counteracting the alkaline reaction of the body tissues.

Briefly tabulated, the technic for smear preparations is as follows:

TECHNIC FOR THE FIXING AND STAINING OF SMEARS.

1 Formalin 40%, 2–5 min.*
2 Wash in water.

Simple Stains.		Differential Stains. Gram's method.	
3 10% aq. gentian-violet.		3 Anilin-gentian-violet, 2 min.	
4 Wash water.	4 Blot, dry.	4 Iodine solution, 2 min.	
5 Dry, mount balsam.	5 Sat. bichloride-alcohol, 5 sec.*	5 Alcohol, 95% decolorize.	
	6 Dilute aq. eosin or fuchsin, 5 sec.*	6 Eosin alcohol.	6 Aq. fuchsin (dilute).
	7 Blot, dry, mount in balsam.	7 Oil of origanum.	7 Wash water.
		8 Balsam mount.	8 Dry, mount in balsam.

THE TECHNIC FOR SMEARS OF SPUTUM.

The demonstration of encapsulated pneumococci in smears of exudates by these formalin methods is thus comparatively simple. The demonstration of encapsulated pneumococci in smears of sputum, however, has proved in my experience another problem. With fresh sputum containing exudate coughed up from the lung good results may often be secured, especially if the more purulent portions of the sputum are selected for examination. But more frequently the organisms fail to show good capsule development, and in the secretions from healthy persons the capsule is even less marked. Apparently saliva and mucus offer poor conditions for capsule formation, and, although the cells coming from the lung exudates may retain their capsules for some time, the organisms growing in the mouth secretions may lose their capsules. To demonstrate capsules on such cells, albumin* in the form of blood serum (Hiss) or egg albumin (Boni) must be added, and thoroughly mixed with the sputum before the smears are made. The mucus in which many of the organisms are imbedded protects them from the albumin, and they fail to show capsules. Often it is only the isolated or exposed cells which show capsules. In the fixation by formalin the mucus also protects the cells from the action of this reagent; longer exposure is therefore required, and after this the preparation should be thoroughly washed in water, or some of the formalin will be retained and precipitate the anilin stain when this is used.

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*Thick smears require longer exposure than thin smears of material containing few cells and little detritus.

The demonstration of capsules on the pneumococci in sputum thus depends primarily upon bringing the bacterial cells into an albuminous environment favorable to the preservation or development of capsules.† This accomplished, the staining procedure is purely a matter of choice. With the formalin preparations the Gram stain may be used and a differentiation for practical purposes at once secured, as on further study in culture, Gram-positive, encapsulated organisms of pneumococcus morphology practically always give the biological characters of the pneumococcus. Occasionally encapsulated forms of the *Micrococcus tetragenus* resemble atypical pneumococci, but usually there is little difficulty in differentiating these forms. The *Streptococcus mucosus*, in my experience, cannot be differentiated morphologically from the pneumococcus. Streptococci, in my experience, have rarely given definite capsules‡ with these formalin methods.

THE TECHNIC FOR SECTIONS OF TISSUES.

Although the fixation of the pneumococcus capsule in smears is simple, in tissues hardened for celloidin sections it is difficult and less certain. The chief difficulty lies in securing sufficient penetration. In the body fluids the formalin is diluted, and it combines with the albuminous material which is coagulated. This protects many of the bacterial cells from the action of the formalin, and the capsules of these cells are not properly fixed, and are thus dissolved by subsequent treatment. These difficulties are often encountered in precise histological work, and are largely eliminated by injecting the tissue with the hardening fluid, or, when this is not feasible, by cutting the material into small bits or thin slices.

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*Blood serum seems to mix with sputum better than egg albumin, but as suggested by Professor F. C. Wood, egg albumin, if it is diluted and made isotonic with NaCl solution, will give practically as good results.

†With some pneumococci capsules are obtained with great difficulty. For complete discussion of the differentiation of the pneumococci the reader is referred to the work of Hiss, Borden, and Knapps.

‡Occasionally a faint, hazy periphery, suggesting a shrunken or degenerated, partially dissolved capsule, was noted.

The lungs are easily injected through the trachea and are quickly hardened in the distended condition. The lesions may then be cut into small pieces and the fixation completed in fresh, strong formalin, the whole process taking from three to five hours. After alcohol dehydration the material is imbedded in celloidin and cut in the usual way. It is important to have thin sections* for microscopical examination; otherwise the encapsulated bacteria lying in the exudate among the cells cannot be easily distinguished. After alcohol dehydration these sections may be fixed on the slide by partially dissolving the celloidin with ether, or alcohol and ether, equal parts. A few seconds in alcohol will then harden the thin film of celloidin covering the section, and after washing in water the preparation is ready to be stained. A variety of staining procedures may be employed;† but the Gram method, by virtue of its differentiation, has proved the most useful. Anilin-gentian-violet stain for two to five minutes, iodine solution one to two minutes, alcohol decolorization, eosin-alcohol counterstain, eosin-oil of origanum to clear, and balsam mounting, are the several steps of the technic. This has rarely failed to give good results, but occasionally the pneumococci, after prolonged exposure, or after exposure to weak or impure formalin solutions, decolorize partially in the alcohol. This technical error, when it occurred, was rectified by using a 5 per cent bichloride-alcohol for the decolorization following the iodine solution, so that the material could be studied, though not so accurately as when the cells were properly fixed. By using strong bichloride-alcohol for decolorization, Gram-negative organisms retained the gentian-violet stain and were demonstrable in the sections.

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*Thin sections are readily secured by painting the surface of the block with dilute celloidin-ether between each stroke of the knife.

†A combination of the Nicolle and Van Gieson methods of staining has given some excellent preparations. After staining in Loeffler alkaline methylene blue, the dye is fixed in the bacterial cells by 10 per cent aqueous tannic acid. This is followed by a partial decolorization in alcohol, counterstaining in van Gieson's strong fuchsin-picric acid solution (Freeborn, *Proc. N. Y. Path. Soc.*, 1893, p. 73), differentiation in picric acid alcohol, clearing in picric acid-oil of origanum, and mounting in balsam. The Gram stain may be substituted for the methylene blue and tannic acid stain, but it is apt to decolorize in the acid alcohol. The picric acid cellular stain is more easily studied than the eosin stain.

The difficulties of staining encapsulated pneumococci in sections of diseased tissues are thus purely technical, similar to those met with in all precise histological work, and with due care easily eliminated. By using the Gram method of staining a practical differential procedure is available for the accurate determination of pneumococci in sections of diseased tissues. This is particularly valuable in the study of pneumonic lesions, where contaminations or secondary infections often occur, and cultural examination fails to reveal the true significance of the bacteria isolated, or the relationship of these organisms to the disease processes.

These methods of studying the encapsulated pneumococcus, uniform in principle, for the most part simple, and adaptable to varying conditions, have been of such value in my studies that I believe they may prove similarly useful to other workers in this field.

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